

Non-isotopic analysis of single strand conformation polymorphism (SSCP) in the exon 13 region of the human dystrophin gene

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Abstract

More than 30% of Duchenne and Becker muscular dystrophy (DMD/BMD) patients have no gross DNA rearrangements like deletions or duplications. The large size of the coding sequence of the dystrophin gene (11 kilobases) complicates systematic identification of point mutations. Recently reported approaches based on genomic DNA or mRNA show that chemical cleavage of mismatches is an effective but time consuming and technically demanding method for the identification of point mutations in the human dystrophin gene. We have used a fast and convenient system consisting of PCR amplification of genomic DNA, non-isotopic SSCP analysis, and direct sequencing of PCR products for the detection of mutations in exon 13 and adjacent intron sequences. Sixty-eight DMD patients without detectable deletions or duplications were analysed, resulting in the identification of a point mutation in the coding sequence and two polymorphisms in the 5' flanking intron. The C to T change of the first nucleotide in the third triplet leads to a stop codon and seems to be the cause of the functional deficiency of the gene product in this patient.

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Around 60 to 70% of patients suffering from Duchenne or Becker muscular dystrophy (DMD or BMD) have deletions or duplications of parts of the dystrophin gene.¹⁻³ The remaining one-third of DMD/BMD cases do not have gross rearrangements of the dystrophin gene. Therefore small structural alterations like point mutations are presumed to be responsible for the defective dystrophin.

Owing to the large size of the coding sequence of the dystrophin gene (11 kilobases,⁴ 79 exons⁵) the systematic identification of point mutations is difficult. Up to now about 10 point mutations have been detected by immunological analysis,⁶ or by chemical cleavage of amplified products of reverse transcribed dystrophin mRNA⁷ or PCR amplified genomic DNA⁸ in combination with sequencing. However, although effective, the chemical cleavage^{9,10} method is technically demanding especially for routine diagnoses. In addition, this approach involves the use of hazardous chemicals and radioactive labelling.

Therefore we turned to another promising method for the detection of sequence variants

in PCR amplified DNA fragments: the single strand conformation polymorphism technique (PCR-SSCP). It is based on the fact that the electrophoretic mobility of single stranded DNA in non-denaturing polyacrylamide gels depends not only on its size but also on its sequence.¹¹⁻¹³ The feasibility of the PCR-SSCP technique for detecting DNA alterations has already been shown for other genetic diseases.¹⁴⁻¹⁶ However, in contrast to most of the published examples, we performed a non-isotopic procedure for the identification of unknown mutations using an extremely sensitive silver staining to visualise double and single stranded DNA.¹⁷⁻¹⁹

In this study we have applied non-isotopic PCR-SSCP analysis coupled with silver staining of non-denaturing polyacrylamide gels to detect sequence heterogeneities in the exon 13 region of the human dystrophin gene. Analysis of the DNA from 68 DMD/BMD patients has identified a mutation generating a termination codon and two polymorphisms in the 5' upstream intron adjacent to exon 13.

Materials and methods

PATIENTS AND DNA ISOLATION

Preparation of DNA from lymphocytes of patients was performed as described previously.²⁰ No abnormalities had been found by genomic PCR of selected exons and Southern blot analysis.

DNA AMPLIFICATION

PCR amplification²¹ of genomic sequences encompassing exon 13 (length 238 bp) was performed using up to 500 ng of genomic DNA, 10 pmol each of primers 13F and 13R,²² 200 µmol/l dNTPs, 5 µl of 10 × *Taq* DNA polymerase buffer, and 2.5 U *Taq* DNA polymerase (Amersham) in a total volume of 50 µl. The reaction mixtures were overlaid with 50 µl paraffin oil (Merck) and 25 cycles of PCR (95°C for one minute, 58°C for one to two minutes, 72°C for one minute) were carried out, followed by a final extension (72°C for 10 minutes) in a Techne PHC-1 thermocycler.

SSCP ANALYSIS

PCR reaction products (0.5 to 1 µl) were mixed with 1 µl of 0.2 mol/l NaCl and 3 µl of deionised formamide/0.2 mol/l EDTA solution. Samples were denatured at 95°C for five minutes, quick chilled on ice, and then loaded

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onto a 5% non-denaturing horizontal polyacrylamide gel containing 90 mmol/l Tris-borate, 2 mmol/l EDTA, pH 8.3, and 10% glycerol. The gel was bound to FMC Gelbond PAG (Biozym). Electrophoresis was performed at 500 V for five to seven hours at 20°C on an adapted TGGE device (Diagen). After electrophoresis the gels were silver stained (Diagen) as follows. The gel was incubated twice with 300 ml of a 10% ethanol/0.5% acetic acid solution for three minutes. After washing with deionised water it was incubated for 10 to 15 minutes with 300 ml of a 0.1% AgNO₃ solution. Then the gel was washed twice again followed by incubation (up to 10 minutes) with a freshly prepared mixture of 1.5% NaOH, 0.01% NaBH₂, and 0.15% formaldehyde. The gel was washed again and then soaked for up to five minutes with a 0.75% NaCO₃ solution. Gels can be sealed in plastic bags or they can be air dried and stored for documentation.

Results

SSCP analysis of PCR amplified exon 13 DNA fragments from 68 patients showed three characteristic banding patterns (fig 1). Pattern A was observed predominantly (for example, D21) with a frequency of about 81%. The less abundant pattern B (for example, D58) and pattern C (for example, D108) had frequencies of 11.76% and 7.35%, respectively (table 1). In addition to the characteristic band pattern types, a very small mobility shift of only the upper single DNA strand was detectable in the sample of patient D64 (fig 1). Sequence analyses comparing band pattern types A, B, and C identified point mutations in the 5' adjacent intron sequence of exon 13 (table 2) at positions nt-72 and nt-67 relative to the first coding nucleotide of the exon, obviously representing sequence polymorphisms.

Direct sequencing of two independently amplified PCR products from patient D64 and a normal control showed a single base change in the first position of the third coding triplet of exon 13. The sequence change C to T (fig 2) creates a stop codon (TAA). Therefore a truncated non-functional dystrophin molecule has to be expected, representing only 14% of the full sized protein. This observation correlates well with the severe clinical picture of the patient. The first symptoms were detectable when the patient started to walk and he became wheelchair bound at the age of 9 years.

Discussion

We amplify exon 13 and parts of the flanking intron of the dystrophin gene routinely as part of the screening for major rearrangements in DMD/BMD patients. So far we have screened 72 males and detected four deletions that include exon 13. The PCR products of the remaining 68 patients without deletions were analysed for sequence alterations by a non-isotopic PCR-SSCP approach consisting of non-denaturing polyacrylamide gel electro-

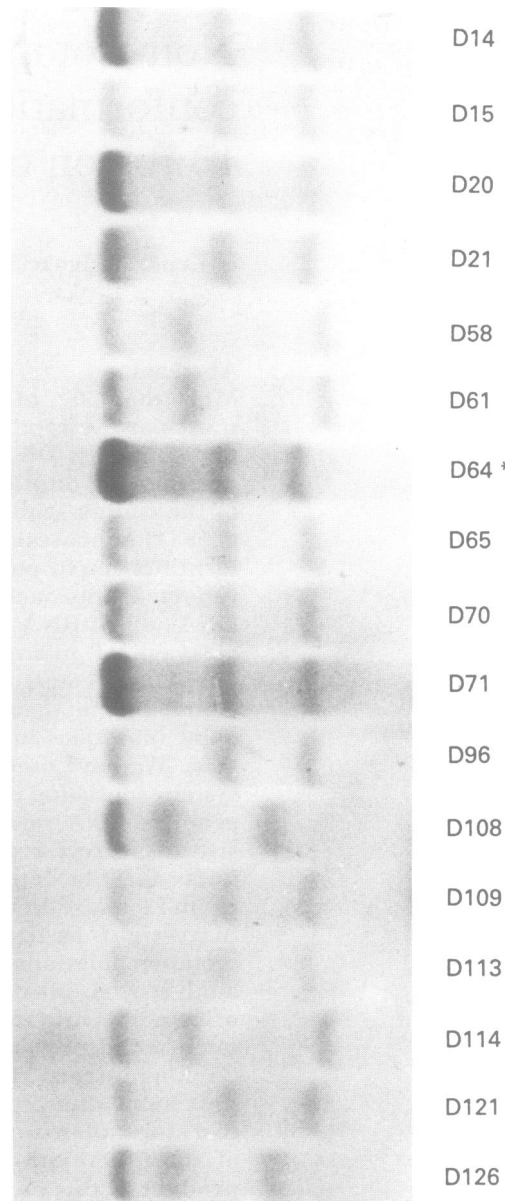


Figure 1 Exon 13 PCR-SSCP analysis of 17 DMD/BMD patients. Electrophoresis was performed at 20°C in a 5% PAA gel containing 10% glycerol for five to seven hours at 500 V. After silver staining (Diagen) we distinguished three different band patterns: the predominant type A (for example, sample D21) and two less abundant patterns, type B (for example, sample D58) and type C (for example, sample D108). The SSCP sample of patient D64, marked with an asterisk, exhibits a very small mobility shift in only the upper single strand whereas the other comigrates with the band of the second strand of the predominant band pattern type A.

Table 1 Polymorphisms in exon 13 flanking sequences identified by non-isotopic SSCP analysis.

Location (relative to the first nucleotide of exon 13)	Sequence change	Frequency
nt-67	A to T	5/68 = 7.35%
nt-72	T to C	8/68 = 11.76%

Table 2 Polymorphisms and the deficiency mutation (1587 C to T) in exon 13 and in the 5' flanking intron sequence. The PCR primer sequence (13F) and the mutant stop codon of patient D64 are underlined. Intron sequences are given in lower case letters and exon sequences in capitals. Nucleotide exchanges are indicated below the corresponding position in the reference sequence and the positions relative to the first coding nucleotide of exon 13 are marked above the sequence tracts.

		nt-72 nt-67	
<u>aataqqaqta</u>	<u>cctgaqatgt</u>	<u>aqcagaaata</u>	aatttcacca
			tttgagagca
			c
			t
aatcatttca		acacacatgt	aagaatatca
		ttttaatttc	ctttaaaca
		nt 7	
ttttatcttt	cagGTGCTTC	AAGAAGATCT	AGAACAAGAA
		T AA	CAAGTCAGGG

phoresis and a sensitive silver staining procedure.

In our study we have detected one point mutation which generates a termination codon causing a loss of more than 85% of the dystrophin coding sequence. In addition, two sequence polymorphisms with a frequency of 11.76% and 7.35%, respectively, were identified in the 5' flanking intron. In a recent study Nigro *et al.*²³ reported a polymorphism in the exon 13 region, but since sequence data were not available in this paper we were unable to compare our two polymorphisms with the reported one.

Different techniques are used in RNA or DNA approaches for the detection of the 30 to 40% of DMD/BMD causing mutations representing no gross gene rearrangements: chemical cleavage of amplified products of reverse transcribed dystrophin mRNA⁷ or PCR amplified genomic DNA⁸ and radioactive multiplex PCR-SSCP.²³

Despite the technical difficulties the RNA approach offers the advantage that several exons can be analysed simultaneously, but in cases of exon skipping the analysis has to be extended to the genomic level in order to identify the presumed splice site mutation which frequently occurs in the flanking intron sequence. In addition, because of the extreme sensitivity of RT-PCR, there is always the risk of amplifying rare, aberrantly spliced products from the ectopic dystrophin transcripts,²⁴ complicating the identification of any corresponding mutation.

For the dystrophin gene, the genomic SSCP approach requires extensive efforts for PCR amplification and gel analysis. However, when all sequence data are available for one subject all 79 exons, including splice site sequences in flanking introns, could be amplified in a single

microtitre plate. The capacity of our electrophoresis devices would allow 79 exons plus available reference samples to be analysed within two or three days. When the technical and experimental prerequisites are well established for the genomic approach by PCR-SSCP it should be possible to perform a complete screening of one patient within a week, which is comparable to the RNA approach using chemical cleavage.

An increasing number of studies dealing with the search for sequence variants or point mutations in human genes have used PCR-SSCP approaches, both radioactive and non-isotopic.^{25,26} Although this method is limited to relatively small DNA fragments, and although in rare cases some mutations may not be detectable under certain conditions of electrophoresis,²⁷⁻²⁹ PCR-SSCP has become more important and widely applied. Therefore we used PCR-SSCP and found it fast, easy to handle, and reproducible. We preferred the non-isotopic method because of the very sensitive visibility of the smallest migration shifts in relatively short gel runs (three to four hours) which might not be detectable in radioactive approaches in cases of strong signals. Owing to possible interfering effects of single stranded DNA fragments when two or more fragments of relatively small size differences are analysed in a multiplex gel run (especially under different conditions of electrophoresis), we only analysed single amplified exons. This avoids possible masking of very slight migration shifts.

Chemical cleavage for detecting single base exchanges seems to be a relatively effective approach. While in an RNA approach Roberts *et al.*⁷ detected mutations in all seven patients analysed, Kilimann *et al.*,⁸ performing a study with amplified genomic DNA, did not observe the exon 13 flanking intron polymorphisms that we found in our study. However, the chemical cleavage method is technically demanding and time consuming, and it involves the use of hazardous chemicals and radioactive labelling. In addition, the labelled probes have a limited applicability because of the half life period of the label, requiring preparation of probes for nearly every new DMD/BMD case in routine diagnosis. Though virtually all mutations can be detected by the well established chemical cleavage method, these circumstances might prevent its wider application in clinical and forensic tests.

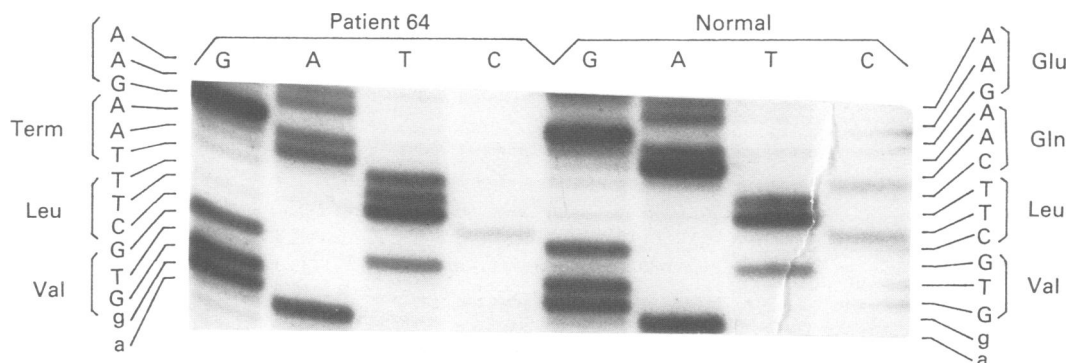


Figure 2 Direct sequencing of the intron-exon boundary of patient D64 compared to a wide type sequence. The mutation is shown as a C to T change in the first position of codon 490 resulting in a termination signal (TAA).

Despite the fact that both approaches discussed have advantages and disadvantages which may result in a general preference of one method considering specific technical and experimental conditions, both chemical cleavage and PCR-SSCP using either RNA or genomic DNA represent equally suitable methods for systematically searching for sequencing variations in the human dystrophin gene.

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